CRYOPRESERVATION OF RABBIT SPERM USING DIMETHYL SULFOXIDE IN COMBINATION WITH TREHALOSE AND HYALURONIC ACID

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ABSTRACT

Rabbit sperm cryopreservation is a great challenge, and there are not a reliable cryoprotectant for the commercial application yet. The present study evaluated the effects of disaccharides (sucrose and trehalose) and hyaluronic acid on rabbit sperm cryopreservation, when were added to a dimethyl sulfoxide (DMSO) containing extender. The result showed that the supplement of 0.05 mol/L trehalose increased the motility ($P < 0.05$), progressive motility ($P < 0.05$), and acrosomal integrity ($P < 0.05$) of frozen sperm after thawing, and 800 μg/mL hyaluronic acid significantly raised the acrosomal integrity ($P < 0.05$). Finally, artificial inseminations were conducted and the fertility rate was 53.9%.

Key words: Dimethyl sulfoxide, Sucrose, Trehalose, Hyaluronic acid**.**

INTRODUCTION

The cryopreservation of rabbit sperm is needed not only by the maximum utilization of genetic resource of good male rabbit, but also the transportation of animals between countries and the preservation of some endangered rabbit breeds (Mocé and Vicente, 2009).

Dimethyl sulfoxide (DMSO) is identified as an effective cryoprotectant (CPA) for rabbit spermatozoa, and disaccharides (*eg*. lactose, sucrose, and trehalose) were considered as non-permeant CPAs for their stabilizing membrane functions during cryopreservation (Dalimata and Graham, 1997). Dalimata and Graham (1997) reported that cmpared with raffinose, trehalose has a significant improvement in rabbit sperm quality, when it was added in an egg yolk-acetamide extender. However, the same CPA may have different effects in different extender backgrouds (Zhang and Li, 2001). In general, the tris-based extender, mainly composed of tris, citric acid, fructose or gluse, is the most frequently used extender in rabbit sperm cryopreservation. whether disaccharides (sucrose and trehalose) have a promoting effect on sperm freezing in the tris extender ramains unknown.

Besides, some macromolecules have also been identified effictive in rabbit sperm cryopreservation, although their exact mechanism is not clear, they might act as non-penetrating cryoprotectants, inducing the formation of ice crystal lattices that may protect sperm from damage. Liu and Wang (2003) found that one of macromolecules, hyaluronic acid, has a protective effect in the cryopreservation of porcine sperm, by increasing the sperm viability and acrosome integrity. However, whether it is valid for rabbit spermatozoa remains unknown.

Thus, one aim of the present study is to evaluate the effects of two disaccharides (sucrose and trehalose) in the sperm cryopreservation based on a tris extender , which contains DMSO. The other aim of this study is to test the effect of hyaluronic acid in trehalose and DMSO containing extender, in order to get an effective compound cryoprotectant.

MATERIALS AND METHODS

Animals and semen collection

Sixty male rabbits at ages from 10 to 12 months (weight about 4.5–5.0 kg) were kept in a single cage and placed in a standard light-dark cycle (light: dark = 16:8). During the period of the experiment, all rabbits were fed ordinary food by specific person and get their drink freely. The rabbits were randomly divided into five groups (twelve rabbit/groups) and one group per day was used for semen collection by turns. The ejaculates of the twelve rabbits were collected separately using prewarmed artificial vaginas. Sperm motility was then examined using the Computer Assisted Sperm Analysis (CASA) system. Only semen with a sperm motility of more than 90% was put into one pool. This experiment lasted for 20 weeks.

Cryopreservation

Part 1 of the experiment, sucrose or trehalose were added into a DMSO-containing tris extender (1 M DMSO, 314 mM tris, 104 mM citric acid, 33 mM glucose, 300 IU/ml of penicillin, and 300 IU/ml of streptomycin, pH 6.8) at a final concentration of 0.05 mol/L, 0.1 mol/L, and 0.25 mol/L respectively, which thereafter was used to dilute the fresh semen at a dilution of 1:1. Every 0.5 ml diluted semen was transferred into a 0.5ml semen straw and was sealed by sealing powder. The straws were put on the surface of a handmade wire mesh for 10 minutes, which was on the top of a thermos cup with liquid nitrogen in. The straws were parallel to and 5 cm over the surface of liquid nitrogen. And then the straws were put in the liquid nitrogen. When thawing, the semen was immersed in a 50°C water bath for 12 seconds, and then incubated in a 37°C water bath for 10 minutes. The motility and progressive motility of sperm were measured using CASA, and acrosomal integrity was determined by staining with Coomassie brilliant blue.

Part 2 of the experiment, different concentrations (400 μg/ml, 800 μg/ml, and 1600 μg/ml) of hyaluronic acid were added into the DMSO containing extender together with 50 mM trehalose. The semen was diluted, frozen and threw as above, and the same indexes were detected.

Artificial insemination (AI)

In order to test the final protective performance of our compound cryoprotectant with 800 μg/ml hyaluronic acid and 50 mM trehalose in the DMSO containing tris extender, AIs were carried out in rabbit farm of Kangda Inc. (Qingdao, China). Two hundred and thirty-three female rabbits with similar weight and age were inseminated with fresh or frozen semen after estrus synchronization in three batches. The synchronized estrus of female rabbit was induced by increasing illumination (80lux for 16 h/day) for 6 days before AI. Every female rabbit was injected with 0.83 μg luteinizing hormone releasing hormone A3 to induce ovulation, and then was injected with 0.5ml thawed frozen semen or fresh semen diluted by tris extender, both of which contain 20 million of motile sperm. The fertility rate, total born, and live born were calculated after farrowing.

Statistical Analysis

The percentages of motile sperm, progressive sperm, and acrosomal integrity were expressed as mean ± SEM, the numbers of born were expressed as mean ± SD. All data were analysed using one-way ANOVA, followed by Bonferroni test. The data of fertility was analysed by Chi-square test. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

In the present study, two kinds of disaccharides, sucrose and trehalose were added into a DMSO containing tris extender, respectively. As shown in figure 1, the supplement of sucrose (0.05 - 0.25 mol/L) did not improve the motility and progressive motility of sperm (Figure 1 a and b, $P > 0.05$). However, 0.05 mol/L trehalose has a significant improvement on the percentage of motile sperm, progressive sperm and acrosome integrity (Figure 1, $P < 0.05$). The research of Rosato and Iaffaldano

(2013) found that freezing medium with BSA plus adequate amounts of disaccharide can improve the cryo-survival of rabbit sperm, but they found BSA/sucrose was more effective than BSA/trehalose. That means the sucrose is more effective than the trehalose in their experiment. However, in our result, trehalose is better than sucrose. The difference probably because we used a different basic extender.

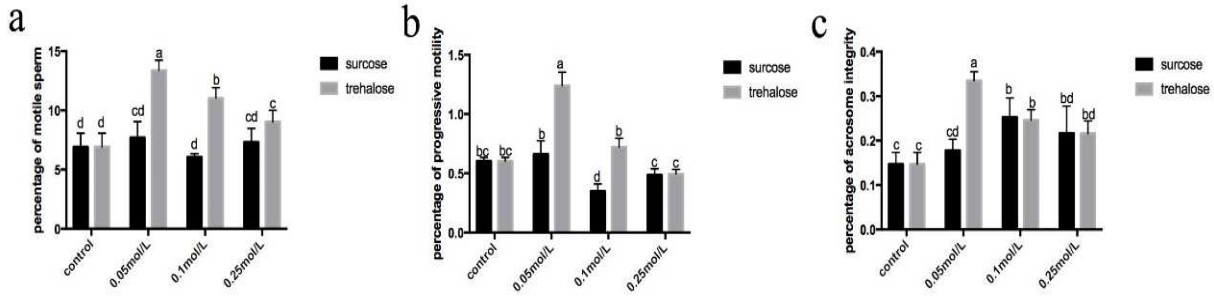

Figure 1: The effect of sucrose and trehalose on motility and acrosomal integrity of cryopreserved/thawed sperm (n=3)

According to the figure 2, the result showed that 800 μg/mL hyaluronic acid has an obvious protective effect on sperm motility and acrosomal integrity compare to control group (Figure 2, $P < 0.05$). 400 μg/mL and 1600 μg/mL hyaluronic acid did not change the percentage of progressive sperm (Figure 2 b, $P > 0.05$), although they raised the percentage of motile sperm (Figure 2 a, $P < 0.05$). This postive effect of hyaluronic acid is consistent with the findings on pig (Liu and Wang, 2003) and human spermatozoa (Sbracia *et al.*, 1997). To our surprise, the acrosome integrity of sperm treated with 400 μg/ml hyaluronic acid was lower than that of control group (Figure 2 c) and it is hard to explain, more samples probably are needed to conform this result in the future.

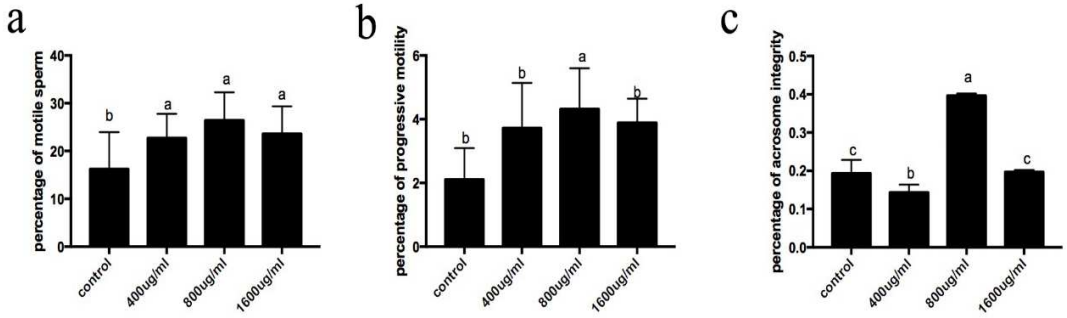

Figure 2: The effect of hyaluronic acid on motility and acrosomal integrity of cryopreserved/thawed sperm (n=3)

As shown in Table 1, the fertility of female rabbits inseminated by frozen semen were 53.9%, the number of total born, and the live born was respectively 5.45±0.536 and 4.78±0.565. This indicates our compound cryoprotectant with 800 μg/ml hyaluronic acid and 50 mM trehalose in the DMSO containing tris extender is effective in the cryopreservation of rabbit sperm, which is qualified to be a cryoprotectant in transportation of animals between countries and the preservation of some endangered rabbit breeds. However, the reproductive performance of frozen sperm were apparently worse than the fresh semen (Table 1). This suggests that it is not practical yet to replace the fresh semen with the frozen semen in rabbit production.

Table 1: Reproductive performance of rabbit does after insemination with fresh semen or frozen semen

Reproductive performance	Semen treatment	
	Fresh	Frozen
% Fertility (N/Total)	80.5 (95/118)[a]	53.9 (62/115)[b]
Total born mean ± SD)	7.87±0.665[a]	5.45±0.536[b]
Live born (mean ± SD)	7.29±0.7[a]	4.78±0.565[b]

Means with different letters on the same row differ significantly (Chi-square test and Bonferroni test)

CONCLUSIONS

In conclusion, the supplement of 0.05 mol/L trehalose and 800 μg/mL hyaluronic acid in a DMSO containing tris extender improves the sperm motility and acrosomal integrity in rabbit sperm cryopreservation.

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